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Functional Distinctions between IMP Dehydrogenase Genes in Providing Mycophenolate Resistance and Guanine Prototrophy to Yeast*

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Abstract

IMP dehydrogenase (IMPDH) catalyzes the rate-limiting step in the *de novo* synthesis of GTP. Yeast with mutations in the transcription elongation machinery are sensitive to inhibitors of this enzyme such as 6-azauracil and mycophenolic acid, at least partly because of their inability to transcriptionally induce IMPDH. To understand the molecular basis of this drug-sensitive phenotype, we have dissected the expression and function of a four-gene family in yeast called *IMD1* through *IMD4*. We show here that these family members are distinct, despite a high degree of amino acid identity between the proteins they encode. Extrachromosomal copies of *IMD1*, *IMD3*, or *IMD4* could not rescue the drug-sensitive phenotype of *IMD2* deletants. When over-expressed, *IMD3* or *IMD4* weakly compensated for deletion of *IMD2*. *IMD1* is transcriptionally silent and bears critical amino acid substitutions compared with *IMD2* that destroy its function, offering strong evidence that it is a pseudogene. The simultaneous deletion of all four *IMD* genes was lethal unless growth media were supplemented with guanine. This suggests that there are no other essential functions of the IMPDH homologs aside from IMP dehydrogenase activity. Although neither *IMD3* nor *IMD4* could confer drug resistance to cells lacking *IMD2*, either alone was sufficient to confer guanine prototrophy. The special function of *IMD2* was provided by its ability to be transcriptionally induced and the probable intrinsic drug resistance of its enzymatic activity.

IMP dehydrogenase (IMPDH)[1] catalyzes the rate-limiting step in *de novo* GTP synthesis. It is an important enzyme whose abundance and activity are correlated with cellular growth rate (1). The enzymology and pharmacology of IMPDH are well studied in mammalian systems because it is a target of chemotherapy, and it plays a critical role in metabolism (2). The IMPDH enzymes across phlya show widely varying sensitivities to mycophenolic acid, with K_i values from 7 nM to 18 μM (3, 4). Also, mutations in human IMPDH may be causally involved in some cases of inherited retinitis pigmentosa (5, 6).

It is becoming clear that duplicated genes are a common feature of many genomes. Members of a duplicated gene family may degenerate into nonfunctional pseudogenes or serve as raw material for evolutionary diversity by acquiring novel functions (7). The human and mouse genomes each contain two IMPDH genes: IMPDH type I is constitutively expressed,

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[1]The abbreviations used are: IMPDH, IMP dehydrogenase; MPA, mycophenolic acid; ORF, open reading frame; GRE, guanine response element; RE, repressive element.

whereas IMPDH type II is inducible (8). *Saccharomyces cerevisiae* contains four IMPDH-like genes (*IMD1–IMD4*; Fig. 1) that display 83–96% amino acid identity between themselves when aligned pairwise. They are closely related to mammalian IMPDHs, showing 58–60% amino acid identity when aligned pairwise with either of the two human proteins. Yeast *IMD1* and *IMD2* appear to be related by an ancient chromosomal duplication (9, 10). *IMD1* may be a pseudogene because it is very close to the telomere, and it contains a frameshifting insertion (Fig. 1; Refs. 9 and 11). It is not known whether *IMD1* is transcribed or can yield a full-length protein *in vivo* as a result of regulated frameshifting. *IMD1* and *IMD2* are the most closely related *IMD* pair (96% amino acid identity) and have proven difficult to delete, presumably due to their telomere-proximal location (Ref. 9; *Saccharomyces* Genome Deletion Project, www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). However, strains were constructed in which a large deletion of chromosome I resulted in the loss of the promoter of *IMD1*, the 5′-untranslated region, and the first 11 codons. This strain and a similar one containing a comparable deletion of part of chromosome VIII and *IMD2* are phenotypically normal, suggesting that neither of these is an essential gene (9). Contrasting results were reported for *IMD2* by Niedenthal *et al.* (12), who indicated that *IMD2* deletion leads to lethality.

Deletion of *IMD3* or *IMD4* does not impact viability (13, 14). Although not located near telomeres, this gene pair is also related through an ancient duplication of portions of chromosomes XII and XIII (10, 15). *IMD4* is the only yeast *IMD* that contains an intron (Fig. 1). Given their high sequence homology and the finding that there are four nonessential *IMD* genes, its seems likely there is functional redundancy among the members of this family, yet the biological and biochemical role of these genes and their gene products in yeast is poorly understood. Whereas redundancy is suggested, there is also specialization between *IMD* genes, as indicated by the ability of *IMD2* to become transcriptionally induced and provide drug resistance (16–18). Although thought to be necessary, it is not known whether induction of *IMD2* is sufficient for the acquisition of drug resistance. Some evidence suggests that *IMD3* may also be induced (19).

IMPDH has also been implicated in transcription elongation. Initially, it was shown that mutation or deletion of *DST1* (also known as *PPR2*), the gene encoding elongation factor SII (also known as TFIIS), can render cells sensitive to 6-azauracil and mycophenolic acid (MPA) (20–23). Both drugs result in the inhibition of IMPDH, which in turn results in a reduction of cellular nucleotide pools (21). Subsequently, mutation of other elements of the yeast elongation machinery has been shown to confer growth sensitivities to MPA and 6-azauracil (24, 25). Transcriptional induction of IMPDH expression has been observed in both mammalian and yeast cells after drug challenge, indicating that nucleotide pool levels are closely monitored *in vivo* and that their biosynthesis can be transcriptionally regulated (16, 17, 26). In yeast, it is likely that *IMD2* contributes the most to this induction. Genetic evidence indicates that an optimally functioning elongation machinery is important for induction of *IMD2* (17–19, 27–29). The inability of yeast with mutations in the transcription elongation machinery to induce *IMD2* explains, in part, their sensitivity to IMPDH antagonists. There is, however, more than one mechanism by which yeast can become sensitive to 6-azauracil or MPA (17, 18, 30).

Multiple factors are involved in de-repression of *IMD2*. Promoter elements governing the response have been identified and shown to confer induction upon heterologous genes (16, 17). The proteins responsible for sensing guanine nucleotide levels and de-repressing or activating transcription are unknown. Although the majority of the inductive response appears to be due to *IMD2*, and deletion of only *IMD2* results in 6-azauracil and MPA sensitivity, there is some indication that other members of this gene family are

transcriptionally active and may also be up-regulated during nucleotide deprivation (17, 19). However, assessment of individual *IMD* transcripts by Northern blot and microarray hybridization techniques is confounded by the high degree of relatedness of their mRNAs. An essential tool in dissecting the differential function of the members of this gene family would be a set of single, double, triple, and quadruple deletions, which would enable the examination of each gene's contribution to IMPDH activity in the cell both before and after induction and the dissection of any functional redundancy.

Here we have constructed such a set of yeast strains and assayed them for growth on rich media and mycophenolic acid. The induction of each member of the *IMD* family was studied in isolation in triple *IMD* mutants. The ability of the different *IMD* genes to rescue the drug-sensitive phenotype was also investigated. The gene family, taken together, is essential, and its loss renders yeast auxotrophic for guanine. By a number of criteria, *IMD1* is functionally inert. We show that *IMD2* and, to a lesser extent, *IMD4* are induced in the presence of these drugs. *IMD3* and *IMD4* are clearly functional because either is sufficient for guanine prototrophy, and both are distinct from *IMD2* because their overexpression by two different means fails to provide drug resistance. The data indicate that *IMD2* encodes an inducible and relatively drug-resistant IMPDH.

MATERIALS AND METHODS

Chemicals

Guanine (Sigma) was dissolved to 50 mM in 1N NaOH, and 250 μl were spread onto 25 ml of solid medium. Mycophenolic acid (Sigma) was dissolved to 15 mg/ml (w/v) in dimethyl sulfoxide and added to molten media before plates were poured. Zymolyase was obtained from US Biological. G418 sulfate (Cellgro) was dissolved in 0.1 M HEPES to 200 mg/ml and used at 250 μg/ml. SC and YPD media have been described previously (31).

Plasmid Construction

IMD Expression Vectors—Single copy and high copy plasmids containing the *IMD1*–*IMD4* genes were constructed as follows. For *IMD1*, *IMD3*, and *IMD4*, each gene was amplified by PCR using *Pfu* DNA polymerase (Stratagene), cut with *Xho*I and *Not*I, and inserted into similarly cut pRS426. The primers used were 5′-gactagtgcggccgcgatcggttgaccgcagtatt-3′ and 5′-gactagtctcgagaatgaatcacggaacccaat-3′ for *IMD1*, 5′-gactagtgcggccgcagggctaggatatcgggaaa-3′ and 5′-gactagtctcgagcggagaaaaagccacaactg-3′ for *IMD3*, and 5′-gactagtgcggccgcctcttaaaggttccgcctca-3′ and 5′-gactagtctcgagccgccttcactagacgaact-3′ for *IMD4*. A similarly constructed *IMD2* plasmid has been described previously (18). The respective plasmids (called pIMD1-Pfu, pIMD2-S288C, pIMD3-Pfu, and pIMD4-Pfu) were used as a source of *Xho*I-*Not*I fragments to move the *IMD* genes to the similarly cut CEN plasmid, pRS316 (pIMD1/316, pRS316-IMD2, pIMD3/316, and pIMD4/316). The pIMD1/316 plasmid was mutagenized (Gene Dynamics, Limited Liability Corp.) by deletion of an adenylate residue 1119 bp downstream from the adenylate of the initiating ATG to create pIMD1-dA/316. The corresponding *Xho*I-*Not*I fragment was removed from this plasmid and inserted into pRS426 to generate the high copy plasmid, pIMD1-dA/426. For expression of the *IMD* genes from the *IMD2* promoter, DNA encoding the open reading frames (ORFs) was digested with *Spe*I and *Xho*I after amplification from the respective plasmids described above using 5′-gcactagtatggccgccattagagactacaagacc-3′ and 5′-gactagtctcgagaatgaatcacggaacccaat-3′ (*IMD1* and *IMD1-dA),* 5′-gcactagtatggccgccattagagactacaagacc-3′ and 5′-gactagtctcgagtcgtaaacataacaccccatca-3′ (*IMD2*), 5′-gcactagtatggccgccgttagagactacaagact-3′ and 5′-gactagtctcgagcggagaaaaagccacaactg-3′ (*IMD3*), and 5′-

gcactagtatgagtgctgctccattggattacaaa-3′ and 5′-gactagtctcgagccgccttcactagacgaact-3′ (*IMD4)* and inserted with the *IMD2* promoter (cut with *Not*I and *Spe*I after amplification from pIMD2-S288C using 5′-gactagtgcggccgcatcggttgagcgcgatatta-3′ and 5′-gcactagttgcttttgctacttgtggagt-3′) into *Not*I- and *Xho*I-cut pRS316. These plasmids are called p2P/IMD1/316 through p2P/IMD4/316.

Promoter deletion derivatives were made by amplifying portions of *IMD2* from pIMD2-S288C and inserting them into pRS426. pIMD2-GRE,RE was generated by inserting *Spe*I- and *Xho*I-cut PCR product made with 5′-ggactagtaactgtcataaacgcattttgt-3′ and 5′-gactagtctcgagtcgtaaacataacaccccatca-3′ into similarly cut pRS426. pIMD2-GRE was created by first amplifying *IMD2* sequence using 5′-ggactagttggtaaaaatttcggctgga-3′ and 5′-gactagtctcgagtcgtaaacataacaccccatca-3′, cutting the product with *Spe*I and *Xho*I, and inserting it into similarly cut pRS426. This intermediate construct was cut with *Not*I and *Spe*I and ligated to a similarly cut PCR product amplified from pIMD2-S288C using 5′-gactagtgcggccgcatcggttgagcgcgatatt-3′ and 5′-ggactagtggcggttacgaaaaccaata-3′. pIMD2-RE was made by cutting pIMD2-GRE,RE with *Not*I and *Spe*I and inserting similarly cut PCR product made from pIMD2-S288C using 5′-gactagtgcggccgcatcggttgagcgcgatatt-3′ and 5′-ggactagtggaatagaatagaatacgg-3′. This family of plasmids was then used to move the corresponding *Xho*I-*Not*I restriction fragments into pRS316 for the final vectors used in Fig. 3.

IMD1-IMD2 Chimeric ORF Constructs—The 550-bp *Bsp*HI-*Spe*I fragment from p2P/IMD1-dA/316 and the 1170-bp *Bsp*HI-*Xho*I fragment from p2P/IMD2/316 were ligated into the plasmid backbone fragment of *Spe*I- and *Xho*I-cut p2P/IMD2/316 to generate p1-dA/548/2. The 550-bp *Bsp*HI-*Spe*I fragment from p2P/IMD2/316 and the 1170-bp *Bsp*HI-*Xho*I fragment from p2P/IMD1-dA/316 were ligated into the plasmid backbone fragment of *Spe*I- and *Xho*I-cut p2P/IMD2/316 to generate p2/548/1-dA. The 315-bp *Spe*I-*Ava*II fragment from p2/IMD1-dA/316 and the 1370-bp *Ava*II-*Xho*I fragment from p2P/IMD2/316 were ligated into the plasmid backbone fragment of *Spe*I- and *Xho*I-cut p2P/IMD2/316 to generate pRSChiA. pRSChiB was constructed by li-gating the 315-bp *Spe*I-*Ava*II fragment from p2P/IMD2/316 and the 1370-bp *Ava*II-*Xho*I fragment from p1-dA/548/2 into the plasmid backbone fragment of *Spe*I- and *Xho*I-cut p2P/IMD2/316. p2P/IMD2/316-G93D and p2P/IMD2/316-T38A were generated by site-directed mutagenesis (Gene Dynamics, Limited Liability Corp.) by changing the Gly^{93} codon and the Thr^{38} codon of p2P/IMD2/316 to GAT and GCT, respectively. pRS316-IMD2-D229N was made by changing the Asp^{229} codon to AAT in pRS316-*IMD2*.

IMD Knockout Vectors—pUC119-IMD2KO was created by inserting three PCR products into the pUC119 vector in separate subclonings. The first insert was a 1720-bp PCR product amplified from S288C genomic DNA using 5′-atcgatgagctcttgcaaaccatcagtgaagc-3′ and 5′-accggtcccgggtgcttttgctacttgtggagtt-3′. The PCR product was digested with *Sac*I and *Xma*I and ligated into pUC119 cut with the same enzymes. The second product was a 2196-bp PCR product amplified from *LEU2* in pRS315 using 5′-gacgtccccgggggtgatgacggtgaaaacct-3′ and 5′-gtatactctagaggcgcctgattcaagaaata-3′. The product was digested with *Xma*I and *Xba*I and ligated into the above plasmid cut similarly. Finally, a 1566-bp *Xba*I- and *Pst*I-cut PCR product amplified from S288C genomic DNA using 5′-atcgactctagatgtccgtatgtcctcctgtt-3′ and 5′-acgggtctgcaggtccccttcaagcacaacat-3′ was inserted into the resulting plasmid from the previous step. This results in a plasmid containing a disruption cassette with *LEU2* flanked by *IMD2* DNA upstream and downstream of the *IMD2* ORF. The *Sac*I and *Pst*I product released from this plasmid was used for homologous recombination and knockout of *IMD2* after lithium acetate transformation and selection on leucine dropout medium.

pBS-IMD1KO was created in a similar fashion to pUC119-IMD2KO. The first insert ligated into pBluescript was a *Sac*I- and *Sma*I-digested 970-bp PCR product amplified from S288C genomic DNA using 5′-ggatccgagctcaacgtcagcagcgtcagtaa-3′ and 5′-aagcttcccgggttgctttcgctacttgtgga-3′. The second insert was an *Sma*I- and *EcoR*I-cut 1280-bp product amplified from S288C genomic DNA using 5′-atcgatcccgggacggtcctgttccctagcat-3′ and 5′-gtatacgaattcgcctcgttcagaatgacacgta-3′. The final insert was an *EcoR*I- and *Xho*I-cut 583-bp product amplified from S288C genomic DNA using the primers 5′-cctagggaattcggcacctgtacatactgcgtta-3′ and 5′-gtatacctcgagcatctatcccctgcccaata-3′. A 2.8-kb knockout cassette released after *Sac*I-*Xho*I digestion was used to transform yeast. The expected DNA sequences were confirmed for all constructs.

Yeast Strains

The yeast strains used in these studies are listed in Table I. DNA transformation was performed as described previously (32). DY868 was constructed by transforming a diploid heterozygous *IMD3* knockout strain (BY4743-26035; Research Genetics) with the *IMD1* gene disruption cassette, selecting for HIS^+ transformants and assaying for integration by PCR using a primer complementary to *IMD1* and a primer complementary to *HIS3*. Strain DY871 was generated from DY868 after transformation with the *IMD2* disruption cassette, selection on leucine dropout medium, and confirmation of accurate deletion of *IMD2* by PCR using a primer complementary to *IMD2* and a primer complementary to *LEU2*. DY872 was generated from DY871 transformed with an *IMD4* gene disruption cassette made by PCR using 5′-accaattccatagctttgaagaaacctaacaaacattttacgatggattcggtaatctccgaaca-3′ and 5′-ttatatgcaaaaataaacttttaaatatctatggatgcttactcaacaccgcagggtaataactg-3′ to amplify *URA3* from the plasmid pRS316. DY872 was sporulated in sporulation medium (31), and tetrads were plated and dissected on guanine-containing YPD. Haploids DY873-DY887 were tested for growth on the respective media, and genotypes were confirmed by PCR.

PCR

PCR conditions were performed as follows: 95 °C for 6 min; followed by 30 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min 30 s; concluded by 8 min at 72 °C. The primers used for diagnosing the intact or deleted state of target genes were as follows: *IMD1*-intact, 5′-tgccatcacctctcgtgata-3′ and 5′-gactagtctcgagaatgaatcacggaacccaat-3′; *IMD1-HIS3* integration, 5′-ggtacgttccacaaggtgct-3′ and 5′-ctagggctttctgctctgtca-3′; *IMD2*-intact, 5′-tggtgcttctattgggactatggac-3′ and 5′-cgctatcggaaactttcattt-3′; *IMD2-LEU2* integration, 5′-tcgtgatttcttggcgcaat-3′ and 5′-gctctgatgccgcatagtta-3′; *IMD3*-intact, 5′-ttggtatgggttcaggctct-3′ and 5′-gactagtctcgagcggagaaaaagccacaactg-3′; *IMD3-Kan*MX4 integration, 5′-gactagtgcggccgcagggctaggatatcgggaaa-3′ and 5′-tgtacgggcgacagtcacat-3′; *IMD4*-intact, 5′-cagttactggcatcaagggta-3′ and 5′-gactagtctcgagccgccttcactagacgaact-3′; and *IMD4-URA3* integration, 5′-ggagcacagacttagattgg-3′ and 5′-atgagctggaaaacgacacc-3′.

Northern Analysis

Cells were grown in liquid media, collected in the logarithmic growth phase, washed once with water, and frozen. Total RNA was isolated from thawed cell pellets by hot phenol extraction and quantitated by measuring absorbance at 260 nm. Total RNA (15 μg) was resolved on a 1% (w/v) agarose gel and blotted onto Zeta-probe GT nylon (Bio-Rad). Filters were baked at 80 °C for 2 h and then prehybridized for a minimum of 3 h at 42 °C in 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt's solution, 50% (v/v) formamide, 1% (w/v) SDS, and 100 μg/ml salmon sperm DNA. Filters were hybridized under the same conditions with $\approx 10^8$ cpm of ^{32}P-labeled DNA probe for 15–18 h. The filters were washed twice at 22 °C in 2× SSC/0.1% SDS for 5 min each and twice in 0.2× SSC, 0.1% SDS for 5 min each, followed by two washes in 0.2× SSC, 0.1% SDS at 42 °C for 20 min each. Washed filters were exposed to Kodak X-Omat film and quantitated with a Fuji

BAS1000 imaging system. The *IMD1* probe was made using pIMD1/426 as template and the primers 5′-acgtttgtcaatttgctaacca-3′ and 5′-gactagtctcgagaatgaatcacggaacccaat-3′. The *IMD2* probe was amplified from S288C genomic DNA using 5′-gtggtatgttggccggtactaccg-3′ and 5′-tcagttatgtaaacgcttttcgta-3′. The *IMD3* probe was amplified from pIMD3/426 using 5′- acgtttgtcaatttgctaacca-3′ and 5′-gactagtctcgagcggagaaaaagccacaactg-3′. *IMD4* probe was amplified from pIMD4/426 using 5′-acgtttgtcaatttgctaacca-3′ and 5′-gactagtctcgagccgccttcactagacgaact-3′. Probes were labeled to a specific activity of $\approx 10^7$-10^8 cpm/μg with Klenow DNA polymerase (Promega, Madison, WI), random hexamer primers (Invitrogen), and [α-^{32}P]dATP (Amersham Biosciences).

RESULTS

To discern the specific role of each yeast *IMD*, it would be ideal to delete the other three copies and study each in isolation. This would provide unequivocal evidence, for example, of whether *IMD1* was transcriptionally active in its natural location and whether *IMD2* was the only family member that responds to nucleotide pool changes. It was also of interest to generate the quadruple deletant to test for viability and for use as an *IMD* null that could be reconstituted.

Starting with a diploid yeast strain heterozygous for an *IMD3* deletion (Research Genetics), we deleted one allele of each of the remaining three *IMD* genes by homologous recombination, thereby generating a strain with one intact and one deleted version of each *IMD*. The resulting diploid was sporulated, and all possible combinations of *IMD* deletions were recovered (four single deletants, six double deletants, four triple deletants, and one quadruple deletant) based upon their ability to grow on the selective media representing each integrated marker. The genotypes were confirmed by PCR (data not shown). These 15 haploid deletion strains were tested for guanine auxotrophy by assaying for growth on rich medium lacking guanine (Fig. 2). Because the quadruple deletant was unable to grow, we conclude that the family of *IMD* genes, taken as a group, is essential for yeast viability. In support of the prediction that *IMD1* is a pseudogene, the *IMD2-IMD3-IMD4* triple deletant also failed to grow, demonstrating that *IMD1* is functionally inert in its natural chromosomal context.

In organisms with a salvage synthesis pathway, the loss of *de novo* synthesis of guanine nucleotides can be bypassed by providing guanine in the growth media. The quadruple mutant, as well as the *IMD2-IMD3-IMD4* triple deletant, showed wild type growth when the medium was supplemented with guanine (Fig. 2). Neither adenine nor hypoxanthine could rescue growth of the quadruple deletant or the *IMD2-IMD3-IMD4* deletant (data not shown). Supplementation with xanthine, the product of IMPDH activity, rescued growth of both strains (data not shown). Hence, loss of the *IMD* gene family rendered cells auxotrophic for guanine. *IMD2*, *IMD3*, or *IMD4* rescued growth in the absence of guanine; *i.e.* each was sufficient to support guanine prototrophy.

We also tested the ability of each deletant to grow in the presence of MPA (Fig. 3). All strains lacking *IMD2* were sensitive to MPA. (Because the quadruple deletant and the *IMD2-IMD3-IMD4* triple deletant cannot grow without guanine supplementation, and guanine circumvents the drug treatment, these two strains were not informative in this assay.) No combination of other deletions, including the simultaneous deletion of *IMD1*, *IMD3*, and *IMD4*, generated such a phenotype. This formally proved that *IMD2* is not functionally equivalent to either *IMD1*, *IMD3*, or *IMD4* or even to all three of these *IMD* genes together.

The availability of all four triple deletants provided an opportunity for us to test the transcriptional induction of each *IMD* in isolation. Cells were grown in liquid media, and RNA was analyzed by Northern blotting at varying times after drug challenge (Fig. 4). Northern blots were probed individually with each of the four *IMD* genes to ensure that differing hybridization efficiencies did not bias the detection of any family member's mRNA. *IMD2* and, to a small extent, *IMD4* were induced by mycophenolic acid treatment (Fig. 4, *Probe: IMD2*, *lanes 7–9* and *Probe: IMD4*, *lanes 4–6*, respectively). Transcript derived from *IMD1* was undetectable (Fig. 4, *Probe: IMD1*, *lanes 10–12*). Levels of *IMD3* mRNA remained fairly constant throughout the treatment (Fig. 4, *Probe: IMD3*, *lanes 13–15*).

We used the drug-sensitive phenotype of an *IMD2* deletion strain as an assay to test *IMD* function. Plasmids bearing *IMD1*, *IMD2*, *IMD3*, or *IMD4* and a "reshifted" version of *IMD1*, in which a single adenylate residue was deleted to generate a full-length *IMD1* reading frame, were introduced into cells. As seen previously (18), the reintroduction of plasmid-borne *IMD2* restored growth on MPA to strains lacking *IMD2* (Fig. 5*A*, *single copy*). No other *IMD* could restore resistance (Fig. 5*A*).

We next asked whether the unique ability of *IMD2* to rescue drug resistance resided in the inducibility of its promoter. We cloned the respective ORFs downstream of the *IMD2* promoter and transformed the plasmids into cells lacking *IMD2*. As expected, episomal *IMD2* efficiently restored growth (Fig. 5*B*). Interestingly, *IMD3* and *IMD4* partially rescued growth when expression of these genes was under control of the *IMD2* promoter. Neither *IMD1* nor frameshifted *IMD1* was effective in restoring growth in equivalent experiments. We confirmed by Northern blot analysis that MPA resulted in transcriptional induction of the each family member under control of the *IMD2* promoter (data not shown). This suggested that part, but not all, of the ability of *IMD2* to confer MPA resistance was due to transcriptional induction and a concomitant increase in IMPDH enzyme abundance. If so, we reasoned that overexpression of *IMD3* or *IMD4* by placing these genes with their natural promoters on high copy plasmids might phenocopy the observations seen in Fig. 5*B*. When present on high copy plasmids, *IMD3* and *IMD4* again provided partial relief from drug inhibition (Fig. 5*C*). We conclude that during drug challenge, the *IMD2* gene product is both quantitatively and qualitatively unique among the family of IMPDHs in yeast because part of the cellular response involves making more IMPDH enzyme, and even when overexpressed, *IMD3* and *IMD4* were only partially active in providing function.

Prior studies have identified regions of the *IMD2* promoter that govern the *IMD2* transcriptional response in reporter as-says (16, 18). These include a GRE ≈300 bp upstream of the transcription start site and a RE surrounding the transcription start site (Fig. 6). To test whether these elements were important for cell growth and drug resistance, we introduced into cells plasmids with derivatives of the *IMD2* promoter driving transcription of the *IMD2* ORF (Fig. 6). A copy of *IMD2* lacking all promoter sequences upstream of the transcription start site did not confer MPA resistance (Fig. 6, *pIMD2-GRE,RE*). A construct lacking the GRE but containing the RE was virtually inactive (Fig. 6, *pIMD2-GRE*). A construct deleted for the RE, whose absence de-represses basal levels of *IMD2* transcription but does not affect induction, was fully competent to rescue growth (Fig. 6, *pIMD2-RE*). Hence, the transcriptional control sequences identified in reporter assays demonstrated the expected behavior in the MPA bioassay, confirming the importance of transcriptional induction in the acquisition of drug resistance.

IMD2 and *IMD1* differ by only 20 amino acids. Yet "re-shifted" *IMD1* was completely inactive in these assays, even when it was overexpressed. This suggested that the putative transcriptional silencing of *IMD1* due to its telomere-proximal location is insufficient to

explain its inactivity *in vivo*. Presumably, 1 or more of the 20 amino acid substitutions inactivated the *IMD1* protein. We tested this idea using chimeric constructs made by exchanging restriction fragments between the *IMD1* and *IMD2* ORFs (Fig. 7*A*). These chimeric reading frames were placed downstream of the *IMD2* promoter, introduced into an *IMD2* deletant, and tested for their ability to confer MPA sensitivity to transformants. Substituting the first 184 amino acids of *IMD1* for those of *IMD2* inactivated the ability of *IMD2* to provide drug resistance (Fig. 7*A*, *construct 3*; Fig. 7*B*). Substituting the carboxyl-terminal 339 amino acids of *IMD2* with those from *IMD1* also inactivated *IMD2* (Fig. 7*A*, *construct 4*). Hence, there are substitutions in both the amino-terminal third and carboxyl terminal two-thirds of *IMD1* that contribute to its inactivity. The 184 amino-terminal residues of *IMD1* were independently introduced into *IMD2* in two segments, as residues 1–106 or residues 107–184 (Fig. 7*A*, *constructs 5* and *6*, respectively). Substitution of the amino-terminal 106 amino acids of *IMD1* completely inactivated *IMD2* (Fig. 7*B*, *construct 5*). Exchanging the segment from 107–184 severely compromised its function (Fig. 7*B*, *construct 6*). The latter finding indicates there is yet a third change in the *IMD1* sequence that negatively impacts its function in this assay. The region from 1–106 contains three amino acid differences between *IMD1* and *IMD2*. At two of these positions, *IMD1* varies from the three other *IMD* genes that have the same amino acid. We independently engineered both substitutions into the reading frame of *IMD2* by site-directed mutagenesis to test whether either was sufficient to inactivate *IMD2* (Fig. 7*A*). The G93D substitution (Fig. 7, *A* and *B construct 8*) completely inactivated *IMD2*, whereas the T38A change (*construct 7*) had little effect. Thus, we have identified one of the amino acid changes that accounts for the biological inactivity of *IMD1*. The reciprocal change (D93G) introduced into *IMD1* did not reactivate it (data not shown). Taken together, these data indicate that *IMD1* possesses numerous amino acid substitutions that inactivate its ORF.

Recent reports have described point mutations in a human *IMPDH* that may be causally involved in the inheritance of retinitis pigmentosa (5, 6). One such mutation is a substitution of asparagine for an aspartate that is conserved between human *IMPDH1*, *IMPDH2*, and all four yeast *IMD* genes. This change was predicted to be highly deleterious to IMPDH function (5). The mycophenolic acid sensitivity assay allowed us to test whether the comparable D → N change in yeast *IMD2* compromised its function. The D229N change did not reduce the ability of *IMD2* to provide guanine auxotrophy (data not shown) or confer MPA resistance to yeast (Fig. 7*B*).

DISCUSSION

IMPDH enzymology and pharmacology have been studied extensively in mammalian cells, partly because of the effective drug therapies directed against this enzyme and the protein's relationship to cell growth and proliferation. Prior studies of the basis for drug sensitivity in yeast were complicated because of the presence of four IMPDH homologs. With four IMPDH homologs, the gene family in yeast is the largest observed thus far. The problem of evaluating functional redundancy and sub- functionalization within gene families is one that is becoming increasingly important as organismal genome projects reveal large numbers of duplicated genes (33). Multigene families also complicate the interpretation of data from microarray and other transcriptome monitoring assays.

The molecular genetic tools available in yeast have allowed us to show that the biological function of the members of the yeast IMPDH gene family is not equivalent. We learned the following: 1) as a group, the *IMD* family is essential, 2) *IMD1* is a pseudogene, 3) no other *IMD* genes can compensate for the loss of *IMD2*, even when they are expressed at high levels, 4) the unique function of *IMD2* stems from the fact that it is transcriptionally inducible *and* its primary sequence confers upon it a role in drug resistance, and 5) *IMD3*

and *IMD4*, although unable to provide drug resistance, are functional family members because cells containing either of these genes alone can survive in the absence of guanine supplementation.

Loss of all four *IMD* genes is lethal unless guanine is provided for salvage synthesis. Because growth on guanine appears normal in the absence of all four *IMD* genes, it seems unlikely that the gene products provide another essential function. Although it is known that yeast possess a functional salvage pathway for synthesis of guanine nucleotides (34, 35), our data are formal proof that salvage synthesis alone is sufficient to sustain cell viability. The relative balance between these pathways is presumably a function of the availability of guanine in the environment.

IMD1 was suspected to be a pseudogene because of a frameshift in the ORF as well as its telomere-proximal location. It is not unusual for disabled or pseudogene variants of a gene family to reside at telomere-proximal positions (11). One idea is that gene family members at subtelomeric locations may fall in and out of usage, providing diversity and changes in copy number that are evolutionarily important (11). Because frameshifting and variegation in the extent of silencing at telomeres (telomere position effect) are found in yeast, and some disabled ORFs are transcribed (11, 36), it was formally possible that *IMD1* was expressed. In fact, the promoter region is very similar to that of *IMD2* (≈90% in the 300 bp upstream of the start codon), including possession of the important guanine response element (16, 18). By a number of criteria, we have demonstrated that *IMD1* is a pseudogene. First, it lacks both of the biological functions possessed by the other *IMD* genes (guanine prototrophy and mycophenolic acid resistance). Secondly, it possesses at least three inactivating amino acid changes. Third, it does not give rise to significant amounts of mRNA, a conclusion that can only be reached after deletion of the other family members because of cross-hybridization between *IMD* sequences. This is consistent with reporter assays showing that the promoter region of *IMD1* is relatively inactive (16).

The ability of mutated *IMD* genes to complement the drug sensitivity of *IMD2* deletants put us in a position to test whether human IMPDH mutations involved in a form of auto-somal dominant retinitis pigmentosa compromise biological function of the cognate yeast enzyme. Bowne *et al.* (5) showed that a substitution of asparagine for aspartate at position 226 in human IMPDH1 segregated with disease in at least three families and was absent from normal individuals, leading them to conclude that this change is causally involved in disease. However, this residue is not required for the enzymatic activity of *Escherichia coli* or human IMPDH2 *in vitro* (37, 38). Our data are consistent with this latter finding because we observed wild type levels of complementation by a version of yeast *IMD2* into which the human mutation was engineered. Because the mutation is thought to cause a dominant genetic disease, we speculate that, if causal, the mutant human IMPDH either interferes with wild type molecules or has acquired a new function that is deleterious to the affected retinal cell type. Expression of the mutant form of yeast *IMD2* in wild type yeast did not significantly compromise drug resistance of a wild type *IMD2* (data not shown).

Although the mammalian *IMPDH* type II gene and yeast *IMD2* are both inducible, their disruptions yield distinct phenotypes. Loss of *IMPDH II* does not result in auxotrophy because its loss affects survival even when guanine is provided to mice through their drinking water (39). The more complex development of a metazoan may preclude survival when only salvage synthesis of guanine nucleotides is possible. Yet, like the yeast *IMD2-IMD3* (or *IMD2-IMD4*) gene pair, there is evidence that mouse *IMPDH I* and *IMPDH II* have non-overlapping biological functions (39, 40). The human *IMPDH1* gene shows additional complexity in alternative promoter usage and gives rise to a variety of transcripts in different tissues (40).

Using a set of *IMD* deletants, we show directly that *IMD2* is functionally unique and confirm a report indicating that *IMD2* is not essential (9). We extend prior work showing that deletion of *IMD2* alone, but not deletion of *IMD1*, *IMD3*, or *IMD4*, conferred mycophenolic acid sensitivity upon yeast (18) and that overexpression of neither *IMD3* nor *IMD4* conferred strong resistance to this drug in wild type cells (30). Here we observe that overexpression of *IMD3* or *IMD4* partially rescues the drug sensitivity of *IMD2* deletants, leading us to conclude that at least two factors lead to the unique function of *IMD2*: its change in abundance due to transcriptional induction, and its amino acid differences relative to *IMD3* and *IMD4*. Indeed, a specific promoter element described in reporter assays (16, 18) is necessary for the role of *IMD2* in drug resistance. Curiously, this element is present in all four genes, suggesting that it is not sufficient for transcriptional induction in the chromosomal context. Weak but inducible transcription from *IMD1* could be elicited when it was plasmid-borne, indicating that its natural proximity to a telomere silences transcription (data not shown). The drug-resistant properties of *IMD2* likely result from one or more of the amino acid positions in which *IMD2* differs from *IMD3* and *IMD4*. It is known that IMPDH enzymes from different species have sensitivities to MPA that vary over 4 orders of magnitude (3, 4). We speculate that the Imd2 protein is intrinsically less sensitive (has a higher K_i) to mycophenolic acid than Imd3 or Imd4.

To unequivocally demonstrate which *IMD* genes were inducible, we generated a collection of triple deletants containing only a single *IMD*. Whereas the transcript of *IMD4* was slightly inducible, that of *IMD3* was not (Fig. 4). This contrasts with the observed increase in Imd3 enzyme seen in response to mycophenolic acid using a proteomic approach (19). The difference could be reconciled if *IMD4* mRNA was under translational regulation, or if its product showed reduced degradation rates in the presence of drug. From our Northern blots, it appears that *IMD3* and *IMD4* contribute the most to constitutive levels of IMPDH, whereas *IMD2* is responsive to changes in intracellular guanine.

Interestingly, the natural chromosomal copies of *IMD3* or *IMD4* were sufficient to prevent guanine auxotrophy, providing strong evidence that they are active IMPDH enzymes. These two genes appear redundant in that they are functionally similar, aside from a slight difference in inducibility. Cells with an uninducible version of *IMD2* whose promoter lacks its GRE are also guanine prototrophs (data not shown). In other words, *IMD2* contains the activities observed in *IMD3* and *IMD4* but has gained (or *IMD3* and *IMD4* have lost) an additional function, drug resistance. Perhaps *S. cerevisiae* contains drug-resistant *IMD2* because it shares a common ancestry with *Penicillium brevicompactum*, which makes mycophenolic acid and may encode a relatively resistant IMPDH. Alternatively, the Imd2 enzyme may have evolved into a relatively drug-resistant enzyme after *IMD* gene duplication and following speciation between the two ascomycetes.

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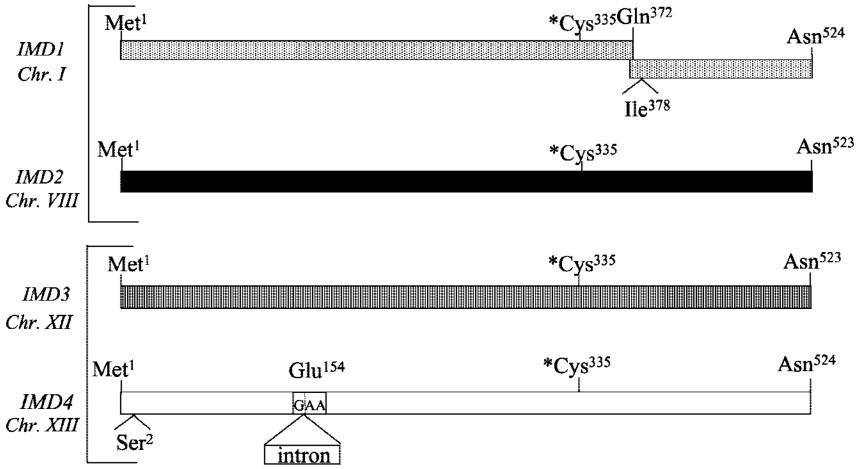

% AMINO ACID IDENTITY BETWEEN YEAST IMPDHs

	IMD2	IMD3	IMD4
IMD1	96%	90%	83%
IMD2		93%	84%
IMD3			89%

Fig. 1. Schematic of the IMD gene family of *S. cerevisiae*

The active site cysteine (*) and specific differences between family members are indicated. Sequence identities were determined by pairwise BLAST analysis (41).

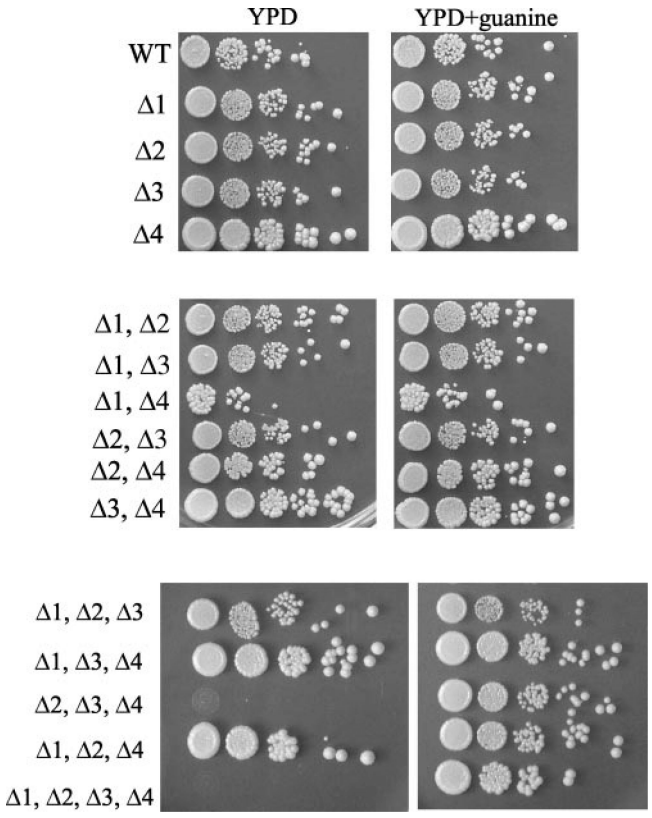

Fig. 2. Guanine auxotrophy of IMD deletion strains

Cells of each strain (Table I) were grown to saturation in 5 ml of YPD with 0.5 mM guanine and diluted to an initial A_{600} of 0.01, followed by five consecutive 10-fold dilutions. Five μl of each dilution were spotted onto YPD plates containing 0.5 mM guanine and 10 mM NaOH or 10 mM NaOH (solvent for guanine) alone. Plates were incubated at 30 °C. Yeast strains used were (reading *down* from the *top row*) BY4741, DY873, DY874, DY875, DY876, DY877, DY878, DY879, DY880, DY881, DY882, DY883, DY884, DY885, DY886, and DY887.

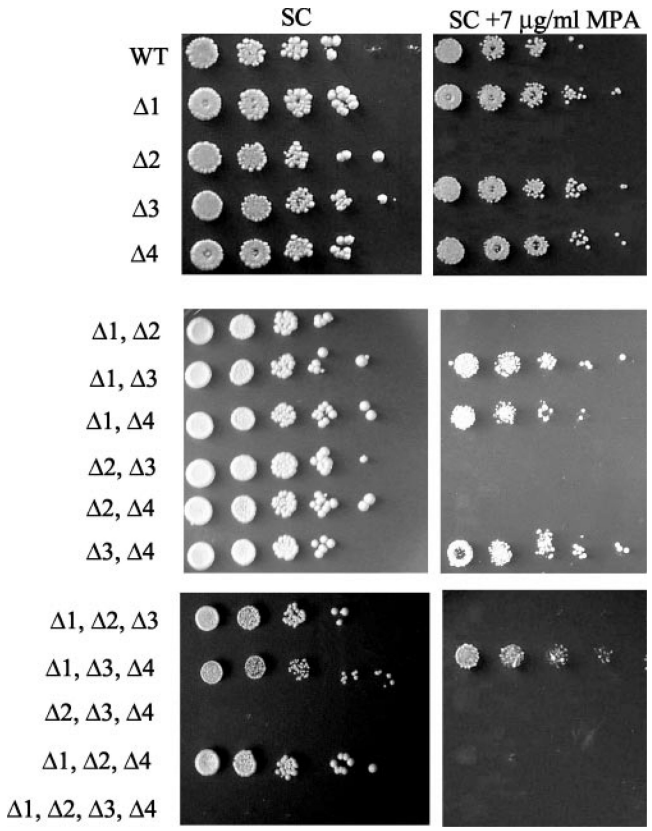

Fig. 3. Mycophenolic acid sensitivity of IMD deletants

Yeast strains were grown overnight in YPD with 0.5 mM guanine and diluted to an A_{600} of 0.01 in SC. Five μl of this suspension and 10-fold serial dilutions thereof were spotted onto SC containing no drug or mycophenolic acid and incubated at 30 °C. Yeast strains used were those indicated in the Fig. 2 legend.

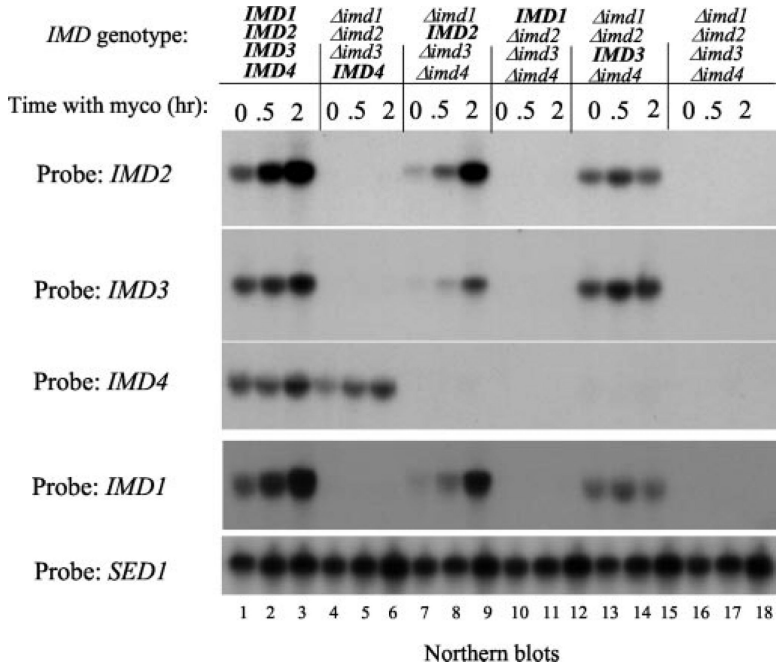

Fig. 4. Inducibility of each IMD

Yeast strains were grown to an A_{600} of ≈0.5 in YPD with 0.5 mM guanine. Cells were pelleted, washed with water, and resuspended in SC to an A_{600} of ≈0.5. Mycophenolic acid (15 μg/ml) was added, and RNA was harvested from aliquots of culture at the indicated times. Northern blots were probed with the indicated *IMD* genes or *SED1* as a control for loading. The strains used were (from *left* to *right*) BY4741, DY883, DY884, DY885, DY886, and DY887.

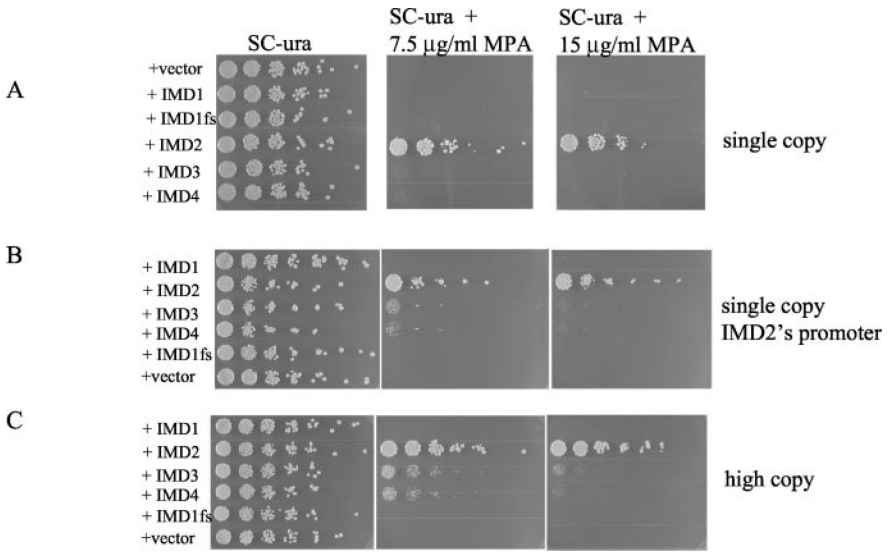

Fig. 5. Functional complementation of IMD2 deletion by expression of yeast IMD family members

Yeast strains lacking IMD2 and transformed with an empty vector or *IMD* genes on a single copy (*A* and *B*) or 2 μ plasmid (*C*) were tested for mycophenolic acid resistance. Strains used (Table I) were (reading *down* from the *top row*) DY731, DY928, DY942, DY929, DY930, DY931, DY980, DY948, DY981, DY950, DY970, DY731, DY917, DY835, DY918, DY919, DY963, and DY964. Cells in logarithmic growth were diluted to an A_{600} of 0.025. Ten μl of this and 4-fold serial dilutions thereof were spotted onto SC medium lacking uracil and containing or lacking 7.5 or 15 μg/ml mycophenolic acid and grown at 30 °C. Strains in *B* contained plasmids in which the respective *IMD* ORFs were driven by the *IMD2* inducible promoter.

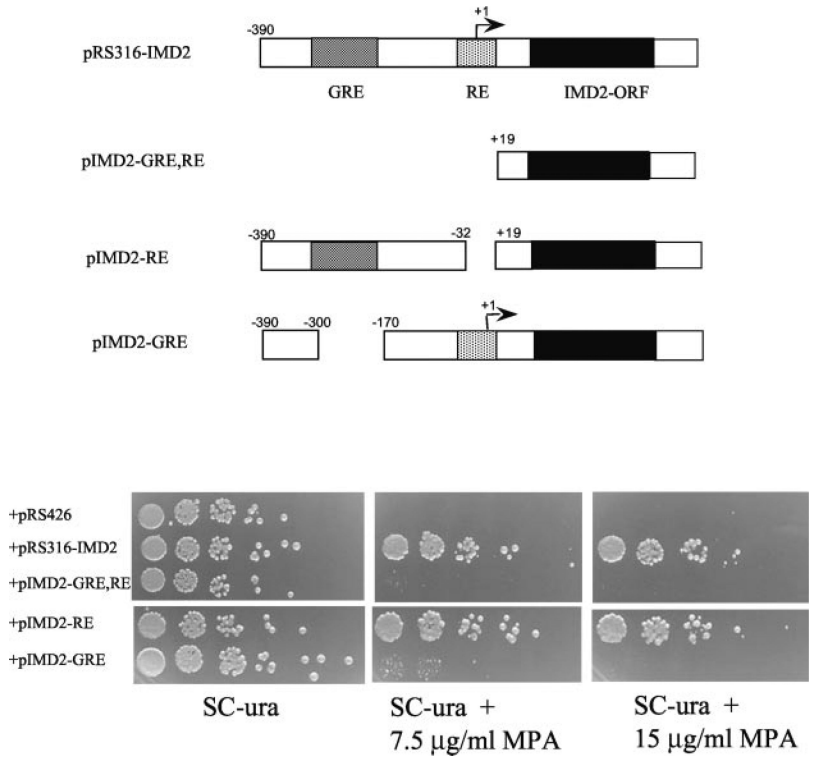

Fig. 6. The guanine response element is required for IMD2 to provide drug resistance

Derivatives of the *IMD2* promoter driving the *IMD2* ORF (*top panels*) were tested for their ability to provide drug resistance when transformed into a yeast strain lacking the *IMD2* gene. Strains DY964, DY929, DY924, DY926, and DY927 (*bottom panels*, reading *down* from the *top row*) were grown in liquid media with or without mycophenolic acid, diluted, and spotted onto solid media as described in the Fig. 2 legend.

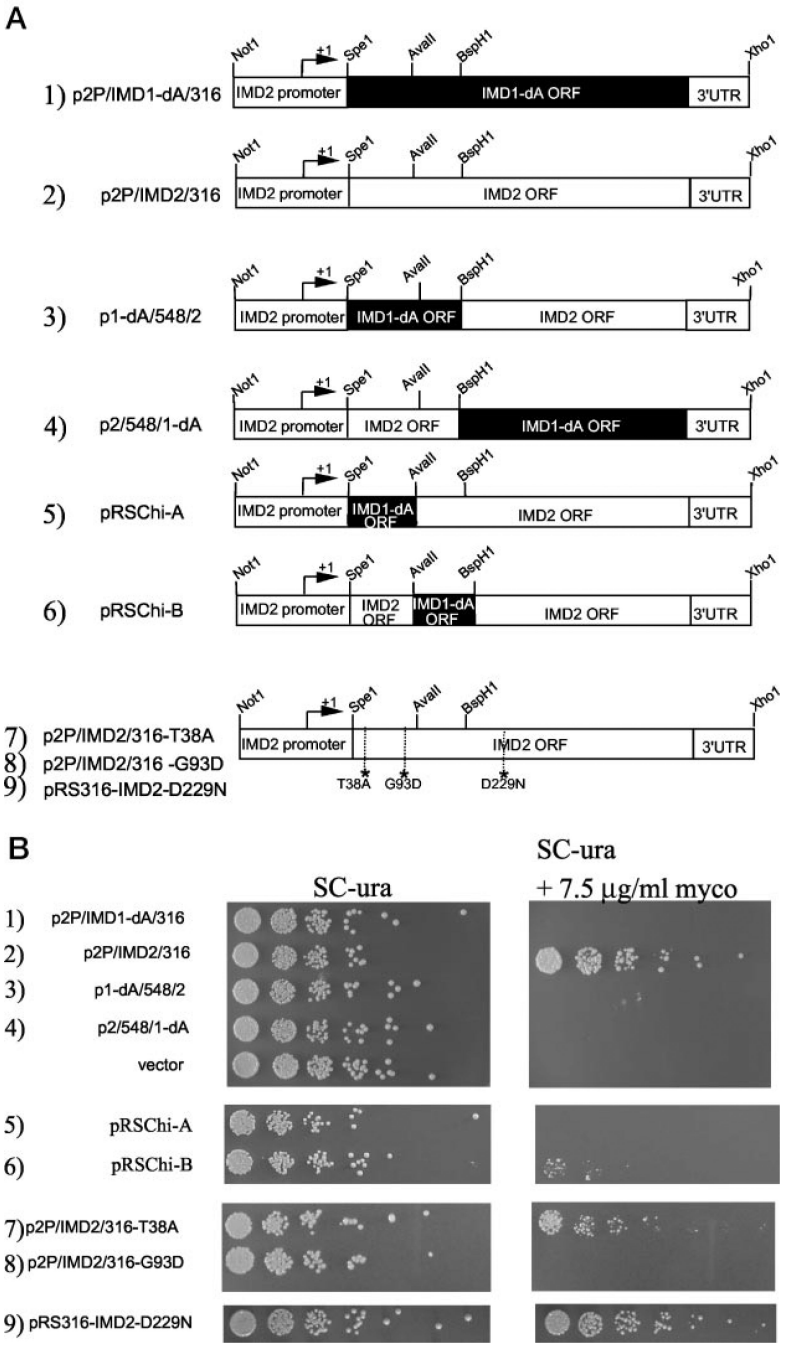

Fig. 7. Testing of mutant IMD2 derivatives for the ability to provide drug resistance to a strain lacking IMD2

Chimeric and site-directed mutants of IMD2 (*A*) were transformed into yeast. The resulting strains (Table I) were tested (*B*) for their ability to grow in the presence and absence of mycophenolic acid as described in the Fig. 2 legend. The strains used were (reading *down* from the *top row*) DY970, DY948, DY976, DY977, DY731, DY982, DY983, DY1017, DY1019, and DY985.

Table I

Yeast strains used in this study

Strain	Genotype
ABGG11[a]	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112*
BY4741[b]	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*
BY4743–26035[b]	*MAT***a**/*MATα his3Δ*/*his3Δ1 leu2Δ0*/*leu2Δ0 ura3Δ0*/*ura3Δ0 MET15*/*met15Δ0 LYS2*/*lys2Δ0IMD3*/*Δimd3*::*kanMX4*
DY731	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pRS316 (URA3)]
DY835	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD2-S288C (URA3 2)]
DY868	*MAT***a**/*MATα his3Δ1*/*his3Δ1 leu2Δ0*/*leu2Δ0 ura3Δ0*/*ura3Δ0 MET15*/*met15Δ0 LYS2*/*lys2Δ0 IMD1*/*Δimd1*::*HIS3 IMD3*/*Δimd3*::*kanMX4*
DY871	*MAT***a**/*MATα his3Δ1*/*his3Δ1 leu2Δ0*/*leu2Δ0 ura3Δ0*/*ura3Δ0 MET15*/*met15Δ0 LYS2*/*lys2Δ0 IMD1*/*Δimd1*::*HIS3 IMD2*/*Δimd2*::*LEU2 IMD3*/*Δimd3*::*kanMX4*
DY872	*MAT***a**/*MATα his3Δ1*/*his3Δ1 leu2Δ0*/*leu2Δ0 ura3Δ0*/*ura3Δ0*/*ura3Δ0 MET15*/*met15Δ0 LYS2*/*lys2Δ0 IMD1*/ *Δimd1*::*HIS3 IMD2*/*Δimd2*::*LEU2 IMD3*/*Δimd3*::*kanMX4 IMD4*/*Δimd4*::*URA3*
DY873	*MATα his3Δ1 leu2Δ0 ura3Δ0 Δimd1*::*HIS3*
DY874	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 Δimd2*::*LEU2*
DY875	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 Δimd3*::*kanMX4*
DY876	*MAT***a***his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 Δimd4*::*URA3*
DY877	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 Δimd1*::*HIS3 Δimd2*::*LEU2*
DY878	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 Δimd1*::*HIS3 Δimd3*::*kanMX4*
DY879	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 Δimd1*::*HIS3 Δimd4*::*URA3*
DY880	*MATα his3Δ1 leu2Δ0 ura3Δ0 Δimd2*::*LEU2 Δimd3*::*kanMX4*
DY881	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0Δimd2*::*LEU2Δimd4*::*URA3*
DY882	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 Δimd3*::*kanMX4 Δimd4*::*URA3*
DY883	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 Δimd1*::*HIS3 Δimd2*::*LEU2 Δimd3*::*kanMX4*
DY884	*MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 Δimd1*::*HIS3 Δimd3*::*kanMX4 Δimd4*::*URA3*
DY885	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 Δimd2*::*LEU2 Δimd3*::*kanMX4 Δimd4*::*URA3*
DY886	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 Δimd1*::*HIS3 Δimd2*::*LEU2 Δimd4*::*URA3*
DY887	*MATα his3Δ1 leu2Δ0 ura3Δ0 MET15 lys2Δ0 Δimd1*::*HIS3 Δimd2*::*LEU2 Δimd3*::*kanMX4 Δimd4*::*URA3*
DY917	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD1-Pfu (URA3 2)]
DY918	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD3-Pfu (URA3 2)]
DY919	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD4-Pfu (URA3 2)]
DY924	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD2-GRE,RE (URA3)]
DY926	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD2-RE (URA3)]
DY927	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD2-GRE (URA3)]
DY928	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD1/316 (URA3)]
DY929	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pRS316-IMD2 (URA3)]
DY930	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD3/316 (URA3)]
DY931	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD4/316 (URA3)]
DY942	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD1-dA/316 (URA3)]
DY948	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [p2P/IMD2/316 (URA3)]
DY950	*MATα Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [p2P/IMD4/316 (URA3)]

Strain	Genotype
DY963	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pIMD1-dA/426 (URA3 2)]
DY964	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pRS426 (URA3 2)]
DY970	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [p2P/IMD1-dA/316 (URA3)]
DY976	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [p1-dA/548/2 (URA3)]
DY977	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [p2/548/1-dA (URA3)]
DY980	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [p2P/IMD1/316 (URA3)]
DY981	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [p2P/IMD3/316 (URA3)]
DY982	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pRSChi-A (URA3)]
DY983	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pRSChi-B (URA3)]
DY985	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [pRS316-IMD2-D229N (URA3)]
DY1017	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [p2P/IMD2/316-T38A (URA3)]
DY1019	*MATa Δ4HIS3 trp1⁻ ura3–1 his3–11,15 leu2–3,112* [p2P/IMD2/316-G93D (URA3)]

[a] From A. Barton and D. Kaback (University of Medicine and Dentistry of New Jersey).

[b] From Research Genetics/Invitrogen.